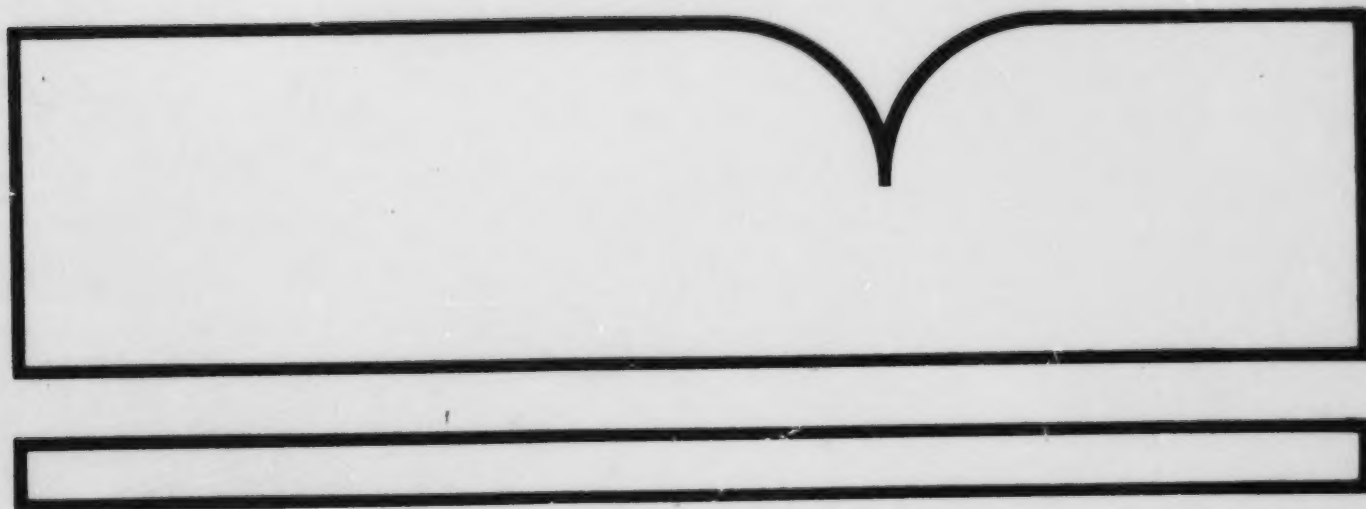


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Mutagenic Effects on Mammalian Cells

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CYCLOPENTA-FUSED ISOMERS OF BENZ[A]ANTHRACENE II:
MUTAGENIC EFFECTS ON MAMMALIAN CELLS

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CYCLOPENTA-FUSED ISOMERS OF BENZ[A]ANTHRACENE II: MUTAGENIC
EFFECTS ON MAMMALIAN CELLS.

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INTRODUCTION

Cyclopenta-fused polycyclic aromatic hydrocarbons (PAH) are a unique class of PAH found in the environment. Acenaphthylene, acephenanthrylene and cyclopenta[cd]pyrene represent characterized cyclopenta-PAH already identified as air pollutants (1,2). The pyrolytic synthesis of PAH from two carbon fragments (3) suggests that many more such cyclopenta-ring fusions are possible and may be characterized from environmental samples. Cyclopenta-PAH are non-alternate PAH in which the fused five membered ring provides a new site for metabolic attack by the cytochrome P-450 mixed-function oxidases. The study of the metabolism, metabolic activation and mutagenesis of these chemicals allows a probe into the mechanism of oxygen transfer and the stereo- and regio-specificity of the cytochrome P-450 mixed-function oxidases as well as an understanding of the competition between sites of metabolic action by these enzymes.

This chapter is a preliminary report of metabolism and mutagenesis studies with four cyclopenta-fused isomers of benz[a]anthracene: benz[j]aceanthrylene, BjA; benz[e]aceanthrylene, BeA; benz[l]aceanthrylene, BlA; and benz[k]acephenanthrylene, BkA. These PAH were prepared by methods previously reported at earlier Battelle PNA symposia (4,5) and recently reported in the Journal of Organic Chemistry (6). The identification of the major microsomal metabolites of these four PAH appears in a companion chapter in this book (7).

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MATERIALS AND METHODS

Cell Culture Conditions

V79 cells were provided by Dr. E. Huberman, Argonne National Laboratory, Chicago, Illinois. The cells were maintained in Williams's medium E supplemented with 10% fetal bovine serum and 2mM L-glutamine. Cultures were incubated in humidified incubators at 37°C in 5% CO₂ in air. Cell cultures were routinely HATG-selected before each experiment.

S9-Mediated Mutagenesis

The V79 cells were plated at 1×10^6 cells/25 cm² T-flask (2 flasks per treatment group) and incubated overnight. The media was removed and the flasks were incubated in 2 ml of the reaction mixture, containing an Aroclor-1254 induced rat liver S9 fraction at the appropriate protein concentration, 0.6 ml Dulbecco's Phosphate Buffered Saline with 0.49 mg MgCl₂·6H₂O per ml, glucose-6-phosphate (13 mg/ml), NADPH (6.3 mg/ml) and PBS containing 5 µg/ml Garamycin, and PAH. The glucose-6-phosphate and NADPH were sterilized by filtration prior to use. The flasks were incubated for 4 hr at 37°C in 5% CO₂ in air. The cells were then washed twice with PBS and refed with 5 ml of fresh media. The control cells were treated in the same manner, except culture media was used instead of reaction mixture. The cells were plated for determination of plating efficiency and mutagenicity. The cells were dispersed using 0.25% trypsin and 0.5 mM EDTA, centrifuged and resuspended in a small volume of media. The V79 cells were counted using a hemocytometer. For determination of plating efficiency, 100 V79 cells were seeded in 5 ml of complete media per 60 mm Petri dish (5 dishes/point) and allowed to grow 6-7 days at which time they were fixed and stained. For 6-thioguanine (6TG) resistance, two 75 cm² T-flasks per group were seeded at 200,000 cells/flask and allowed to grow 4 days. At this time, the cultures were reseeded again at 5×10^5 cells per 75 cm² T-flasks, and on the 7th day following treatment the cultures were replated into 6TG (final concentration 5 µg/ml) containing media at 2×10^4 cells/60 mm dish. Dishes were fixed and stained 12-14 days later without a media change. Plating efficiency was determined as described above.

Irradiated Syrian hamster embryo (SHE) cells (5000 rad) were seeded at 2×10^6 cells/75 cm² T-flask and incubated overnight at 37°C in 5% CO₂ in air. The next morning V79

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cells were seeded on top of the SHE cells at 3×10^5 V79 cells per flask. The V79 cells were allowed to attach to the SHE cells and then treated with the PAH. The treatment period was 48 hours. At this time, the V79 cells were dispersed using 0.25% trypsin and EDTA, centrifuged and resuspended in a small volume of media. Determination of plating efficiency and 6TG resistance was done as described for the S9 mediated mutation assays.

[³H]BjA Metabolism Studies

Metabolism studies with S9 or SHE cells were carried out using the same procedures as described above for the mutation studies except the V79 cells and media were omitted. [³H]BjA, specific activity 100 $\mu\text{Ci}/\mu\text{mol}$ was incubated for the appropriate time and the incubates extracted (after freeze-thawing for the SHE cells) with ethyl acetate-acetone (2:1). The organic extracts were reduced in volume and subjected to HPLC using a 6.2 mm id x 25 cm Zorbax ODS column and a complex gradient system (7) of tetrahydrofuran-water (1:3) and methanol. Water soluble [³H]BjA conjugates which were conjugated to glucuronic acid or sulfate were subjected to enzymatic hydrolysis according to previously published procedures (8) and the hydrolyzed [³H]BjA metabolites analyzed by HPLC. Metabolites were identified by cochromatography with authentic synthetic standards or with metabolites obtained from microsomal incubations and previously identified by spectroscopic analyses.

RESULTS

The SHE cell and S9 mediated V79 cell mutagenesis systems which were used to examine the genetic activity of the cyclopenta-PAH were first evaluated with benzo[a]pyrene (BaP) as a standard (Fig. 1). BaP gave a strong dose related increase in 6TG resistant mutants in the SHE cell system with a plateau at 0.5 $\mu\text{g}/\text{ml}$. In the S9 system the BaP response reached a plateau with 1.0 mg of S9 protein. Similar experiments with SHE cell mediated V79 cell mutagenesis with BjA, BeA, and BkA were negative at concentration ranges of 0-2.0 $\mu\text{g}/\text{ml}$ where significant cytotoxicity was evident at the higher concentrations. BIA was moderately active generating a response of 200 6TG resistant mutants/ 10^6 survivors at 2.0 $\mu\text{g}/\text{ml}$.

A series of experiments with BjA, BeA, BkA, and BIA in the S9 mediated V79 cell system were performed in order to examine both S9 protein (0-3.0 mg/ml) and substrate

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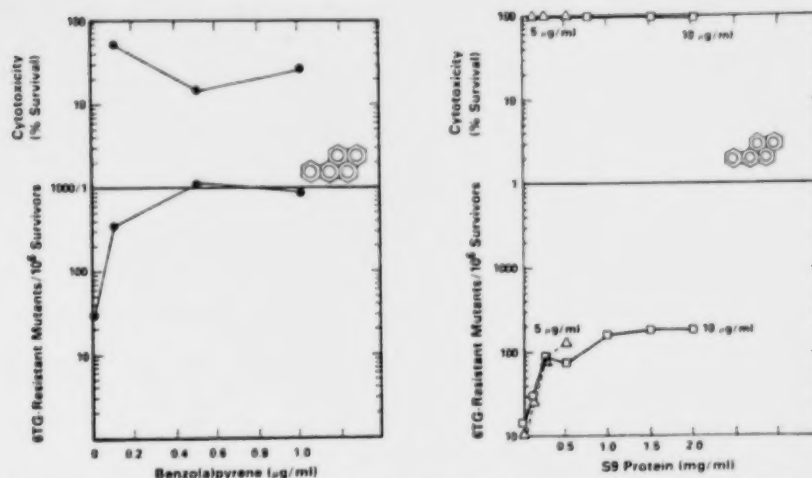


FIGURE 1. SHE cell mediated and rat liver S9 mediated V79 cell mutagenesis by BaP. Left panel: SHE cell mediated mutagenesis as a function of BaP concentration. Right panel: rat liver S9 mediated mutagenesis as a function of S9 protein concentration at 5 and 10 µg/ml BaP.

concentration (1,5,10 µg/ml) responses (Fig. 2,3). BJA and BeA produced significant responses at 5 and 10 µg/ml with maximal mutation frequencies of 350-480 6TG resistant mutants/10⁶ survivors. Both PAH exhibited a plateau of mutagenic activity at 0.5 mg/ml of S9 protein (Fig. 2). BkA was weakly mutagenic in the S9 mediated V79 mammalian cell mutagenesis assay, producing only 85 6TG resistant mutants/10⁶ survivors (Fig.3). However, B1A under the same experimental conditions induced concentration related responses with maximal mutation frequencies of 1000 6TG resistant mutants/10⁶ survivors (Fig. 3). The mutagenic activity of B1A like that of BJA and BeA reached a plateau at an S9 concentration of 0.5 mg/ml.

B1A-1,2-oxide, synthesized by m-chloroperoxybenzoic acid oxidation of the parent hydrocarbon(9), and anti-BaP-7,8-dihydrodiol-9,10-epoxide were examined for mutagenic activity in V79 cells in the absence of an exogenous metabolic activation over a concentration range of 0-2.0 µg/ml (Fig.4). As expected (10) the BaP-diol-epoxide was highly cytotoxic and induced 950 6TG resistant mutants/10⁶ survivors at 0.1 µg/ml. B1A-1,2-oxide also induced a concentration dependent response in V79 cells reaching almost 200 6TG resistant mutants/10⁶ survivors at 1.0 µg/ml.

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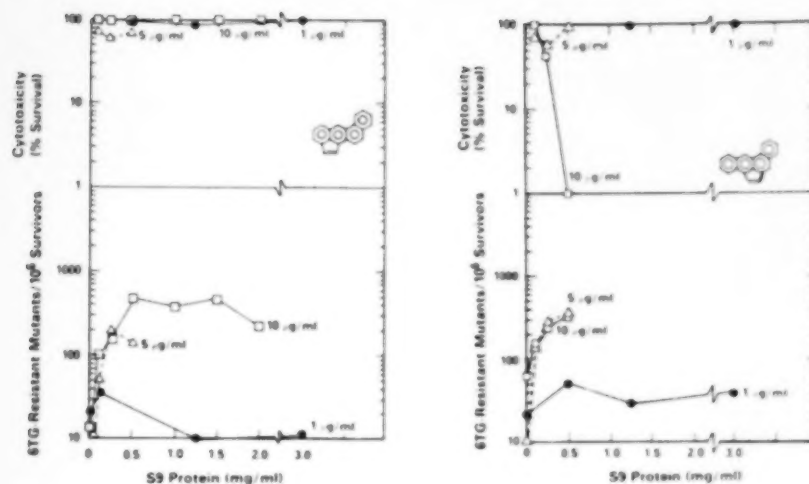


FIGURE 2. Rat liver S9 mediated V79 cell mutagenesis by B[a]A (left panel) and B[a]P (right panel) as a function of substrate and S9 protein concentration.

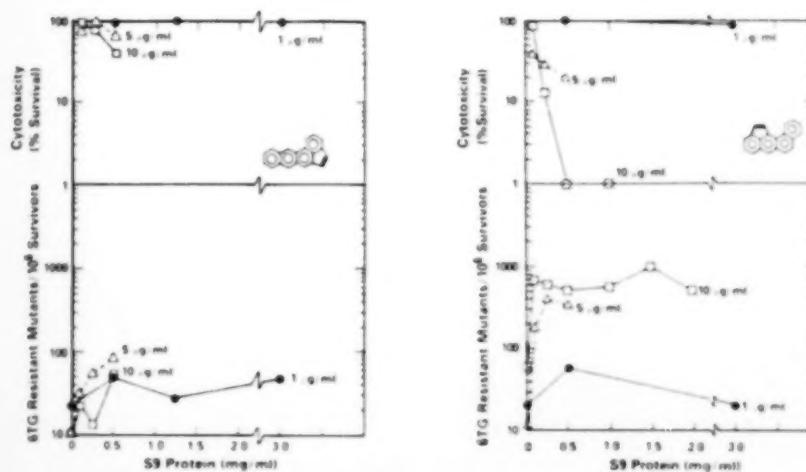


FIGURE 3. Rat liver S9 mediated V79 cell mutagenesis by B[a]K (left panel) and B[a]L (right panel) as a function of substrate and S9 protein concentration.

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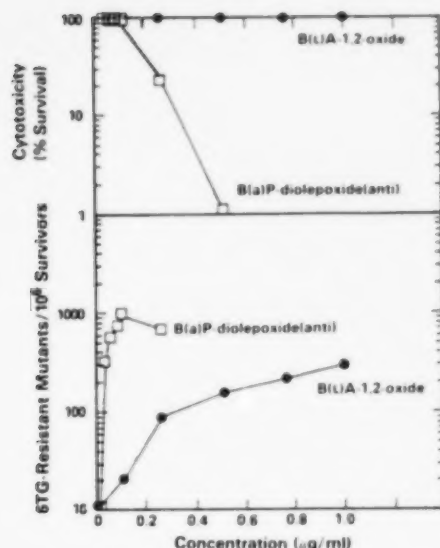


FIGURE 4. Mutagenic effects of B[a]A-1,2-oxide and anti-BaP-7,8-dihydrodiol-9,10-epoxide in V79 cells.

The metabolism of [^3H]B[a]A was studied in irradiated SHE cells and in Aroclor-1254 induced rat liver S9 using HPLC techniques. [^3H]B[a]A (15 µg/ml) was metabolized by rat liver S9 and the disappearance of the substrate was measured over a range of S9 concentrations (0-3.0 mg/ml) after a 15 min incubation (Fig. 5). At 2.0 mg/ml 100% of the substrate was metabolized. The major metabolite formed was B[a]A-1,2-dihydrodiol, with lesser amounts of B[a]A-9,10-dihydrodiol, and B[a]A-11,12-dihydrodiol (7). SHE cells metabolized [^3H]B[a]A (10 µg/ml) to two major products: B[a]A-1,2-dihydrodiol and B[a]A-9,10-dihydrodiol (Fig. 6). B[a]A-9,10-dihydrodiol was isolated in the unconjugated form, while B[a]A-1,2-dihydrodiol was identified as a glucuronic acid and sulfate conjugate as well as in the unconjugated form. The ratio of the total B[a]A-9,10-dihydrodiol metabolites to the total B[a]A-1,2-dihydrodiol metabolites was 2.3. After 48 hr of incubation SHE cells metabolized 57% of the [^3H]B[a]A administered.

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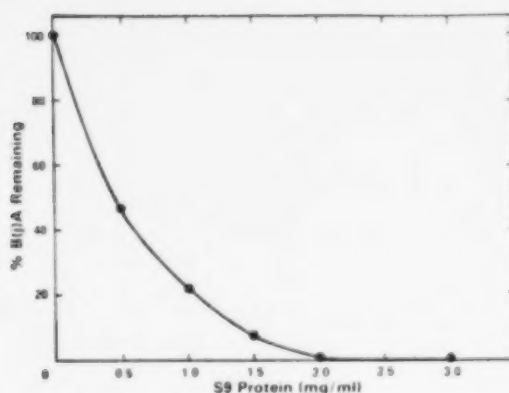


FIGURE 5. Metabolism of BjA by rat liver S9. [^3H]BjA ($15 \mu\text{g/ml}$) was incubated for 15 min. with varying protein concentrations.

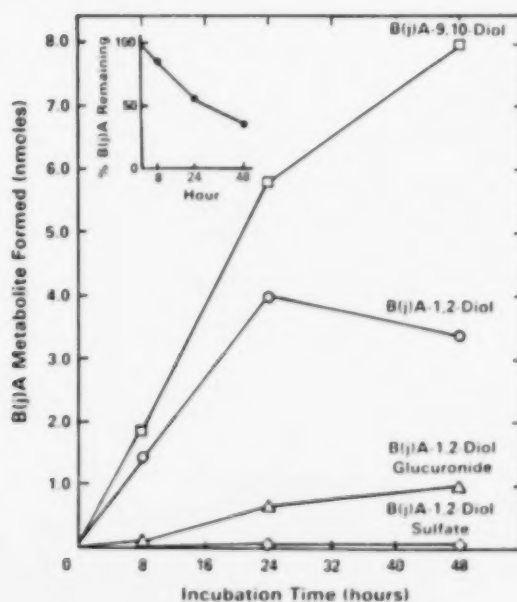


FIGURE 6. Metabolism of BjA by SHE cells. SHE cells (2×10^6 /dish) were treated with [^3H]BjA ($10 \mu\text{g/ml}$) for 8, 12, 24, and 48 hrs. Unconjugated and conjugated metabolites were isolated and identified according to procedures described in materials and methods.

DISCUSSION

The genetic activity of the four possible cyclopenta-fused isomers of benz[a]anthracene has been demonstrated in mammalian cells using alterations at a specific gene mutation locus. All of these unique PAH exhibited mutagenic responses as evidenced by significant increases in the induced mutation frequencies. Each of these PAH produced concentration-responses indicative of a true chemical alteration of DNA. The use of the proper metabolic activation system proved crucial to the exhibition of mutagenic activity. Huberman and coworkers (11) had previously reported that irradiated Syrian hamster embryo fibroblasts were more efficient and effective at activating polycyclic aromatic hydrocarbons to their active genotoxic forms than the standard Aroclor-1254 induced rat liver homogenate (S9). BaP, 7-methylbenz[a]-anthracene, and 7,12-dimethylbenz[a]anthracene, all having structural similarities to the cyclopenta-fused-benz[a]anthracenes were all activated to their genotoxic forms when V79 cells were cocultivated with Syrian hamster embryo fibroblasts (12). However, these fibroblasts were not capable of activating B[a]A, B[a]A, and B[a]A to genotoxic forms. B[a]A was active in the Syrian hamster embryo fibroblast mediated V79 system. In order to understand these differences in metabolic capability, the metabolism of B[a]A was studied under conditions which closely approximated the experimental conditions with which the V79 mutation studies were performed. Syrian hamster embryo fibroblasts were quite capable of metabolizing B[a]A to two major dihydrodiols: B[a]A-1,2-dihydrodiol, and B[a]A-9,10-dihydrodiol. After 48 hr of incubation almost 60% of the B[a]A administered was metabolized. The 9,10-position was favored over the 1,2-position almost 2.3 to 1 when the total unconjugated and conjugated metabolites were considered. When the metabolism of B[a]A was studied in Aroclor-1254 induced rat liver S9 it was determined that the ratio of the two dihydrodiol metabolites was reversed with B[a]A-1,2-dihydrodiol predominating. At concentrations of S9 which produced maximal mutagenic responses, approximately 60% of the B[a]A administered was metabolized after 15 min of incubation. Undoubtedly after 4 hr of incubation, (the incubation time that the S9-mediated-V79 cell experiments were performed) all of the B[a]A would have been metabolized.

The isolation of a dihydrodiol strongly implicates an arene oxide as an obligatory intermediate. Therefore,

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BjA-1,2-oxide is strongly suggested as one of the major arene oxide metabolites which contribute to the overall mutagenic activity of BjA in V79 cells.

In an accompanying paper (7) we described the isolation of the major metabolites of BjA, BeA, BkA and BlA. In each case, the cyclopenta-ring dihydrodiol was the major metabolite or one of the major metabolites formed. To test the hypothesis that cyclopenta-ring oxides are genetically active, the corresponding oxide of BlA; BlA-1,2-oxide was prepared and evaluated in V79 cell for induction of 6-thioguanine resistance. It was found that BlA-1,2-oxide was quite active (Fig. 4) and could account for 50-80% of the mutagenic activity of BlA in the Aroclor-1254 induced rat liver S9 mediated V79 cell system.

We conclude that BjA and BlA exert their genetic activity to a major extent in V79 cells through the formation of the cyclopenta-ring oxides. This is contrasted with the activity of these PAH in Aroclor 1254 induced rat liver S9 mediated Salmonella typhimurium which seems to be almost completely dependent on cyclopenta-ring oxidation (7,13) an observation in accord with previous observations with cyclopenta[cd]pyrene (14-16).

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